Blurring in patients with temporal lobe epilepsy: clinical, high-field imaging and ultrastructural study

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Magnetic resonance imaging-positive temporal lobe atrophy with temporo-polar grey/white matter abnormalities (usually called ‘blurring’) has been frequently reported in patients with temporal lobe epilepsy associated with hippocampal sclerosis. The poor distinction of grey and white matter has been attributed to various causes, including developmental cortical abnormalities, gliosis, myelin alterations, a non-specific increase in temporal lobe water content and metabolic/perfusion alterations. However, there is still no consensus regarding the genesis of these abnormalities and no histopathological proof for a structural nature of magnetic resonance imaging changes. The aim of this study was to investigate the pathological substrate of temporo-polar blurring using different methodological approaches and evaluate the possible clinical significance of the abnormalities. The study involved 32 consecutive patients with medically intractable temporal lobe epilepsy and hippocampal sclerosis who underwent surgery after a comprehensive electroclinical and imaging evaluation. They were divided into two groups on the basis of the presence/absence of temporo-polar blurring. Surgical specimens were examined neuropathologically, and selected samples from both groups underwent high-field 7 T magnetic resonance imaging and ultrastructural studies. At the clinical level, the two groups were significantly different in terms of age at epilepsy onset (earlier in the patients with blurring) and epilepsy duration (longer in the patients with blurring). Blurring was also associated with lower neuropsychological test scores, with a significant relationship to abstract reasoning. On 7 T magnetic resonance image examination, the borders between the grey and white matter were clear in all of the samples, but only those with blurring showed a dishomogeneous signal in the white matter, with patchy areas of hyperintensity mainly in the depth of the white matter. Sections from the patients with blurring that were processed for myelin staining revealed dishomogeneous staining of the white matter, which was confirmed by analyses of the corresponding semi-thin sections. Ultrastructural examinations revealed the presence of axonal degeneration and a significant
Introduction

The findings of studies by Falconer et al. (1964) and Margerison and Corsellis (1966), reinforced by those of recent MRI studies (Bernasconi et al., 2000), suggest that hippocampal sclerosis plays a leading role in the genesis of seizures in patients with temporal lobe epilepsy. Recent reports indicate that hippocampal sclerosis is frequently associated with other pathologies (Blumcke et al., 2000), and those of recent MRI studies (Falconer et al., 2000; Tassi et al., 2009), but a number of studies have shown that even in the absence of a second and independent principal lesion, the abnormalities found in patients with temporal lobe epilepsy go beyond the hippocampus, thus suggesting a more widespread substrate for the generation or persistence of seizures. MRI has revealed ipsilateral temporal atrophy with temporo-polar grey/white matter abnormalities in 32–68% of patients with temporal lobe epilepsy and associated hippocampal sclerosis, which have been described as a loss of grey/white matter demarcation together with increased signal intensity in T2-weighted images (Choi et al., 1999; Meiners et al., 1999; Mitchell et al., 1999, 2003; Coste et al., 2002; Adachi et al., 2006). This type of abnormality (commonly called ‘grey/white matter blurring’) is described in many articles as one of the most sensitive radiological markers of focal cortical dysplasia, but some authors suggest that it may reflect the presence of densely heterotopic neurons in the subcortical white matter or myelin alterations (Hardiman et al., 1988; Choi et al., 1999; Meiners et al., 1999; Thom et al., 2001).

Recent imaging techniques have confirmed that the loss of grey/white matter demarcation in the temporal pole of patients with temporal lobe epilepsy and hippocampal sclerosis is not due to imaging artefacts, thus corroborating the hypothesis that epilepsy may depend on a more widespread temporal lobe disturbance (Meiners et al., 1994, 1999; Mitchell et al., 1999; Moran et al., 2001; Kahane et al., 2002; Bernasconi et al., 2005; Sankar et al., 2008; Concha et al., 2009). The suggestion that extra-hippocampal structures (particularly the temporal pole) may play an important role in patients with temporal lobe epilepsy and hippocampal sclerosis is supported by electroclinical and perfusion data (Ryvlin et al., 1998, 2002; Mitchell et al., 1999; Moran et al., 2001; Chassoux et al., 2004; Chabardes et al., 2005; Weder et al., 2006).

As temporal lobe epilepsy is the most frequent form of drug-resistant focal epilepsy, surgery is an important and safe option, allowing patients to gain seizure freedom (Wiebe et al., 2001), and the increasing numbers of operated subjects with a good postoperative follow-up have also provided specimens for precise neuropathological studies and the investigation of anatomical-clinical correlations. However, there is still no consensus regarding the genesis of these abnormalities, and no definite correlation has yet been found between histopathological findings and MRI-revealed structural abnormalities, with gliosis (Falconer et al., 1964), developmental cortical abnormalities (Hardiman et al., 1988; Thom et al., 2001), myelin loss (Meiners et al., 1999) and a non-specific increase in temporal lobe water content (Mitchell et al., 1999; Concha et al., 2009) all being suggested as possible causes. Furthermore, the question as to whether the grey/white matter abnormalities revealed by MRI are epileptogenic has not been answered, although studies by Coste et al. (2002) and Adachi et al. (2006) argue in favour of an epileptogenic zone involving the side of the abnormalities. There are also conflicting data concerning surgical outcomes: Mitchell et al. (1999) found that the proportion of seizure-free patients was the same in those with and without grey/white matter abnormalities, whereas Choi et al. (1999) found a significantly higher success rate in the patients with abnormalities, although Mitchell’s findings are supported by those of a recent study by Schijns et al. (2011) that examined the impact of temporal lobe grey/white matter abnormalities on postoperative seizure outcome in patients with temporal lobe epilepsy and hippocampal sclerosis, undergoing selective amygdalohippocampectomy or anterior temporal lobectomy. All of these studies were carried out using traditional methods that are routinely available in clinical practice, and the results did not reveal with certainty the aetiopathology of the temporo-polar abnormalities found in a variable proportion of patients with temporal lobe epilepsy.

The aim of this study was to investigate the pathological substrate of the temporo-polar blurring revealed by means of routine clinical 1.5 T MRI using the non-traditional approaches of high-field (7 T) MRI combined with light and electron microscopy of surgical specimens taken from drug-refractory patients with temporal lobe epilepsy and hippocampal sclerosis. The clinical correlations of the abnormalities were also investigated.

Materials and methods

Subjects

The study involved 32 consecutive patients (11 males and 21 females; mean age: 40 ± 9 years, range: 24–60) with medically intractable temporal lobe epilepsy and radiological evidence of hippocampal sclerosis who underwent surgery at the Carlo Besta Neurological Institute in Milan, Italy. Patients with evidence of hippocampal sclerosis associated with other principal lesions (tumours, vascular malformations and scars etc.) were excluded.

Keywords: temporal lobe epilepsy; neuropathology; electron microscopy; high-field MRI; epilepsy surgery
The patients underwent surgery after a comprehensive electroclinical and MRI evaluation; none of them required the invasive presurgical identification of the epileptogenic zone to be removed.

On the basis of the findings of the presurgical 1.5 T MRI evaluation, the patients were divided into two groups: 18 with hippocampal sclerosis plus ipsilateral temporo-polar abnormalities (Group 1) and 14 with hippocampal sclerosis but normal ipsilateral grey/white matter definition in the temporal pole (Group 2). To assess the presence or absence of these abnormalities, all of the magnetic resonance images were independently reviewed by two experienced observers (L.D. and F.V.) blinded to the neuropathological findings, who used the criteria proposed by Colombo et al. (2009) and issued a consensus report.

Preoperative 1.5 T magnetic resonance imaging

All of the MRI studies were performed using the same 1.5 T instrument (Siemens Avanto). The MRI protocol included: transverse spin-echo double-echo images of the entire brain (repetition time: 2000–2500 ms, echo time: 20–90 ms, number of averages: 1, matrix: 128 × 256, field of view: 230 mm, slice thickness: 5 mm); coronal fast spin-echo T2-weighted images (repetition time: 2300 ms, echo time: 100 ms, number of averages: 4, matrix: 256 × 256, field of view: 230 mm, thickness: 3 mm); coronal transverse spin-echo FLAIR images (repetition time: 8000 ms, echo time: 100 ms, inversion time: 2000 ms, number of averages: 3, matrix: 238 × 256, field of view: 230 mm, thickness: 3 mm); and coronal fast spin-echo inversion recovery T1-weighted images (repetition time: 3000 ms, echo time: 20 ms, inversion time: 400 ms, number of averages: 3, matrix: 256 × 256, field of view: 230 mm, thickness: 3 mm). The transversal and coronal sections were acquired parallel and perpendicular, respectively, to the axis of the hippocampal formation.

No contrast medium was used, and all of the T1- and T2-weighted images were qualitatively evaluated by means of careful visual inspection in terms of morphology, the thickness and signal intensity of the cortex of the cerebral hemispheres and the distribution and signal intensity of the white matter. The MRI features of malformed cortical development, atrophic changes and signal abnormalities in the cortex and white matter were recorded. Hippocampal sclerosis was qualitatively demonstrated by means of the comparative visual detection of atrophy and loss of definition of the internal structures of the hippocampal formation and increased signal intensity in the T2-weighted images. Associated abnormalities of mammillary bodies, the fornix and the fimbria were also evaluated. Particularly, attention was given to the interface between the cortex and the underlying white matter, with the definition of the inner margin of cortical ribbon and even mild signal abnormalities from the subcortical white matter being carefully investigated. The morphology and signal intensity of the grey matter and white matter of the temporal poles were evaluated on the coronal sections throughout the anteroposterior extent of the temporal lobe.

Neuropsychological testing

All of the patients underwent a neuropsychological assessment before surgery and 6 months afterwards. Abstract reasoning, immediate and long-term memory and verbal fluency were evaluated using Raven’s Coloured Progressive Matrices, Short Story, Rey’s Complex Figure, Selective Reminding, Corsi Blocks Supraspan and Word Fluency tests (Giovagnoli et al., 2011). The controls were 32 healthy subjects matched in terms of chronological age (mean age: 38.18 ± 11.00 years), years of schooling (mean age: 12.12 ± 3.38 years) and gender (21 males, 11 females).

Surgery and follow-up

All of the resections were performed for strictly therapeutic reasons after the patients had given their informed consent. None of the patients underwent selective amygdalohippocampectomy. The resections were limited to the temporal lobe in all cases, and their extent was planned preoperatively on the basis of the location of the epileptogenic zone (as shown by electroclinical data) and the risk of postsurgical deficits. Seizure freedom was monitored periodically and determined using Engel’s classification (Engel, 1987); only patients with a minimum follow-up of 12 months were considered.

Histology

Immediately after surgery, the resected temporal lobes and hippocampi were fixed in 4% paraformaldehyde buffered solution. After 24 h, the temporal lobe specimens were cut into 4–6 coronal slabs (5 mm thick) throughout its anteroposterior extent and post-fixed in the same solution for 4–5 days. Alternate slabs were embedded in paraffin or in 6% agarose to be cut by a vibratome (VT1000S, Leica). For routine neuropathology, 7-μm thick sections were cut and stained with thionin (0.1%), haematoxylin and eosin, and Klüver-Barrera. Additional serial sections were processed for immunohistochemistry using antibodies against glial fibrillary acidic protein (GFAP; 1:1000 Millipore), non-phosphorylated neurofilaments (SMI 311; 1:250, Sternberger Monoclonals Inc.), neuron-specific nuclear protein (NeuN; 1:1000, Chemicon International), CD68 (1:1000, Dako) and fibrinogen (1:600, Dako). Agarose-embedded slabs were cut into 50-μm thick serial sections, counterstained with thionin and processed using the primary antibodies and an immunoperoxidase procedure described elsewhere (Garbelli et al., 1999).

Quantification of the degree of gliosis and possible inflammatory changes in the white matter of all the samples was performed. Images of sections reacted with the corresponding antibodies; GFAP and CD68, respectively, were captured with a digital camera in randomly placed visual fields using ImageProPlus 6.3 software (Media Cybernetics). Gliosis was evaluated considering the intensity of the colour immunostaining (saturation) in 0.56 mm² for each case, whereas CD68-positive cells density was calculated in 0.56 mm² using a 2D cell-counting technique. The white matter neuronal density was estimated by counting NeuN-positive neurons in the depth of the white matter, at least 500 μm from the grey/white matter boundary (Blumcke et al., 2011), and by two different approaches: a 2D cell-counting technique performed on all samples on representative 2.2 mm² and, according to the Optical Fractionator technique (West et al., 1991), with use of the StereoInvestigator software (MicroBrightField) in the subgroup of the patients submitted to ultrastructural study (see later). The Optical Fractionator is a stereological method for estimating the total number of neurons in a region; the technique combines use of the Optical Disector (a 3D probe for counting neuronal nuclei) and the Fractionator (a systematic random sampling). We considered upper and lower guard zones, corresponding to the surfaces where the tissue is most affected by sectioning, of 3 μm, and the square counting frame in the x-y plane was 150 x 150 μm. The number of frames ranged from 25 to 36. NeuN-positive nuclei that came into focus, either fully inside the counting box or touching the inclusion lines, were counted.
Finally, to evaluate abnormalities in blood vessel and blood–brain barrier function, fibrinogen immunoreactivity in all specimens was performed.

The hippocampi were kept separate and processed for neuropathological diagnosis to confirm the presence of hippocampal sclerosis (the grading is not considered in this report). The slides were independently reviewed by two neuropathologists, one of whom was unaware of the initial diagnoses and clinical data. Any disagreements were discussed until an agreed-upon diagnosis had been reached. The presence of focal cortical dysplasia was diagnosed on the basis of the new International League Against Epilepsy classification system (Blumcke et al., 2011) and classified as type IIa due to the confirmed neuropathological presence of hippocampal sclerosis.

Correlative 7 T magnetic resonance imaging and ultrastructural study

A subgroup of nine patients (four from Group 1 and five from Group 2) underwent a combined high-field MRI and morphological/ultrastructural study. To this end, one slab from each specimen (corresponding to the anterior part of the temporal lobe and embedded in 6% agarose) was examined by means of experimental MRI using a 7 T horizontal-bore scanner (BioSpec 70/30 USR, Bruker) equipped with 200 mT/m gradients and a 35-mm transceiver quadrature coil. High-resolution T2-weighted images were acquired using the parameters: echo time: 50 ms, repetition time: 4.3 s, number of averages: 36, slice thickness: 0.7 mm, field of view: 32 × 32 mm² and data matrix: 256 × 256, which yielded 73 × 73 μm² in-plane resolution. The diffusion tensor imaging acquisitions were carried out using a spin-echo sequence (echo time: 40 ms, repetition time: 5 s, number of averages: 1, slice thickness: 0.7 mm, field of view: 32 × 32 mm², data matrix: 128 × 128) that yielded a 250 × 250 μm² in-plane resolution. The diffusion times were Δ = 22 ms and Δ = 12 ms, and there were five b₀ images, six directions and seven b-values (800, 1500, 2000, 2500, 3000, 3300 and 3500 s/mm²). Mean diffusivity and fractional anisotropy maps were estimated from the raw data images. The FMRIB Software Library (www.fmrib.ox.ac.uk/fsl) was used for the tensorial analysis.

After MRI, the slabs were cut into 50-μm thick serial coronal sections and processed for: (i) staining with 0.1% thionin for structural control and Black-Gold® II (0.3% dissolved in 0.9% saline, Histo-Chem) for myelin visualization; (ii) immunohistochemistry as previously described; and (iii) electron microscopy.

One section from each case (chosen from those stained with thionin and Black-Gold® II) was processed for electron microscopy using a protocol described elsewhere (Garbelli et al., 1999). The osmicated sections of Group 1 revealed uneven staining, and therefore two areas of interest in the deep white matter were excised: one corresponding to a lightly stained (light) zone and one corresponding to an intensely and apparently homogeneously stained (dark) zone. The osmicated sections of Group 2 showed intense and homogeneous white matter staining throughout, and therefore only one area was excised. From each area, 0.5-μm semi-thin sections were cut by means of an ultramicrotome (Reichert–Jung Ultracut E), mounted on glass slides and counterstained with toluidine blue for light microscopy examination. To estimate the number of myelinated fibres in the white matter, semi-thin sections were observed using an oil objective lens (×100, NA 1.32), and two areas of interest from the upper left and two from under right corners were captured, corresponding to a final surface of 18544 μm² for each sample. Afterwards, ultra-thin sections (500 Å) representative of the selected areas were placed on 200-mesh copper grids (EMS), counterstained with lead citrate and uranyl acetate and examined using an EM109 electron microscope (Zeiss). All of the microphotographs were qualitatively evaluated by two independent and experienced observers (M.M. and G.M.) using a non-descriptive numerical code. A modified version of the protocol of Tang et al. (2004) was used for the morphometric analyses. Briefly, thin sections were cut to avoid overlapping bias, and, starting from a random mesh of the grid, the two opposite corners of 10 consecutive fields of each section were photographed at a magnification of ×4400. Empty or damaged fields were discarded. A total of 20 digital images were taken of each area of interest and were analysed by three independent observers (G.Mi., V.M. and G.Ma.) using a semi-automatic program (NIS-Elements BR 3.1 Software, Nikon). The area of each counting frame was 379.21 μm² and the total examined area was 7584.2 μm². The myelinated fibres, on both semi- and ultra-thin sections, were counted when they were completely inside the counting frame or partially inside and only touching the counting lines; they were not counted if they touched the exclusion lines. The glial and microglial cells, present in some microphotographs, were not counted, and their presence did not preclude analysis of a microscopy field. As most of the axons had non-circular profiles, it was not possible to estimate myelin thickness accurately; in these cases, the diameter was measured on the shortest axis of the axonal profile, thus introducing an uncontrolled bias. The evaluated parameters were the mean number of axons, axonal density, circularity, elongation, the circumference and the extra-axonal area. The average parameters of all of the microphotographs of each patient were used for the statistical analysis.

Statistical analysis

Mean age at epilepsy onset, the duration of epilepsy, age at surgery and seizure frequency were compared between the two subgroups using a two-tailed t-test following Levene’s test for equality of variances. The remaining electroclinical features and the presence of blurring were analysed using a contingency table and Fisher’s exact test (P-values of < 0.05 were considered significant).

The neuropsychological test scores of the patient and control group were compared using the Mann–Whitney test. The significance limit for seven pairwise comparisons was set at P < 0.007 (Bonferroni’s correction). Separate Wilcoxon tests were used to compare the pre-operative and 6-month follow-up test scores and regression analyses to explore the role played by blurring in determining neuropsychological performance.

Differences in mean values of glosis, neuronal density and CD68-positive cells were evaluated between the two subgroups by two-tailed t-test, whereas differences in the morphometric parameters were assessed by means of one-way ANOVA, followed by individual post hoc comparisons using Sheffe’s test. A P-value of < 0.05 was considered significant. The Shapiro–Wilk test was used to prove normal data distribution. Statistical analyses were performed using SPSS version 17.

Results

Clinical data and follow-up

The main characteristics of the cohort as a whole are shown in Table 1. The patients’ mean age at surgery was 40 years [range: 24–60; standard deviation (SD) 9.3], and the mean duration of epilepsy was 29 years (range: 5–54; SD 14.3). Their mean age at
epilepsy onset was 12.2 years (range: 2–35; SD 8.4), and mean seizure frequency at the time of surgery was 8.1 per month (range: 1–30; SD 8.2). After 12 months of follow-up (range: 12–60), 29 patients were in Engel’s class I and the remaining three in class II. The cohort was divided into two groups on the basis of the presence of blurring in the preoperative magnetic resonance images (18 patients, 56%) or its absence (14 patients, 44%).

There were no significant between-group differences in terms of mean age at surgery ($P=0.31$), mean seizure frequency ($P=0.14$), the presence of febrile seizures ($P=0.93$) or postoperative outcomes ($P=0.11$), whereas the patients in Group 1 had a significantly younger mean age at epilepsy onset ($P=0.004$) and significantly longer mean duration of epilepsy ($P=0.004$).

A review of the seizure semiology and the video-EEG data showed that the only significant difference between the two groups was the presence of contralateral dystonia during seizures, which was observed in 89% of the patients of the Group 1 and 36% of those in Group 2 ($P=0.028$) (Table 1).

### Neuropsychology

With respect to the controls, patients with blurring were significantly impaired at all of the neuropsychological tests, whereas non-blurring patients were significantly impaired in Short Story and Word Fluency on phonemic and semantic cues. Blurring patients also showed lower Raven’s Coloured Progressive Matrices scores than non-blurring patients. The presence of blurring predicted the preoperative score on Raven’s Coloured Progressive Matrices ($r^2 = 0.16, F = 5.16, P = 0.03$) (Table 2). There were no

### Preoperative magnetic resonance imaging

The FLAIR and T2-weighted images of all of the patients in Group 1 (nine left, nine right) showed hyperintensity in the white matter of the temporal pole ipsilateral to the hippocampal sclerosis. The intensity of the white matter signal was similar to that of the grey matter, with a blurred grey/white matter junction in the temporal pole and ipsilateral hypoplasia of the temporal pole in 13 subjects (72%; Figs 1A–G and 2A). In Group 2 (seven left, seven right), normal and homogenous signal intensity was observed throughout the temporal lobe white matter, and the grey/white matter interface was clearly defined (Fig. 2D); only two cases (1.5%) showed ipsilateral hypoplasia of the temporal pole. No signal abnormalities and no ipsilateral atrophy of the fornix, fimbria or mammillary bodies were detected in any of the 32 patients.

Of the nine cases selected for correlative imaging and electron microscopy study, atrophy of the temporal pole ipsilateral to the hippocampal sclerosis was observed in two patients in Group 1 and two in Group 2.
significant postoperative changes and no differences related to the side of the epileptic region in either group.

**Histology**

The diagnosis of hippocampal sclerosis was confirmed in all 32 patients. Architectural abnormalities in the temporal cortex, characterized by the blurred demarcation of the layers (type IIIa focal cortical dysplasia), were diagnosed in 15 of the 18 patients in Group 1 (83%) and 13 of the 14 patients in Group 2 (93%). Moreover, five patients in Group 1 and three in Group 2 showed temporal lobe sclerosis (Thom et al., 2009), a variant of type IIIa focal cortical dysplasia that is characterized by an atypical band of clustered neurons in the outer part of layer 2 and neuronal depletion in layers 2–3 (Supplementary Fig. 1A and B). In all cases, regardless of the presence/absence of blurring, a variable degree of gliosis of the white matter was evident on GFAP immunostaining, and CD68-positive cells were encountered in all the surgical samples diffusely distributed in the white matter and sometimes located around vessels. According to these observations, quantitative analysis of the GFAP staining intensity and CD68-positive cell density in the white matter revealed no differences between the two groups ($P = 0.30$ for GFAP and $P = 0.19$ for CD68, Supplementary Fig. 1C and D). The presence of an

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**Figure 1** Presurgical (1.5 T) magnetic images of a patient with left hippocampal sclerosis plus ipsilateral temporo-polar abnormalities. The coronal $T_2$-weighted (A) and FLAIR (B) images show atrophy and signal hyperintensity in the left hippocampal formation; the axial section (C) also shows atrophy of the temporal pole. Blurring of the ipsilateral temporal pole can be seen in contiguous $T_2$-weighted (D and E) and FLAIR images (F and G); note the signal hyperintensity of the white matter (asterisks) in the left temporal pole, with an ill-defined grey/white matter junction.
Figure 2 Comparison of presurgical 1.5 (A and D) and ex vivo 7 T magnetic resonance images (B and E) of patients with (A and B) and without blurring (D and E). In the $T_2$-weighted images obtained from the surgical samples of both groups, the boundary between the grey matter and white matter was always clear (compare B and E, arrows), whereas signal intensity in the white matter was dishomogeneous and showed patchy areas of hyperintensity (asterisks) only in the samples taken from patients with blurring. The fractional anisotropy maps show lower values in the white matter of the patients (compare C and F), thus suggesting the loss of white matter integrity.

Table 2 Preoperative neuropsychological test scores in patients and controls

<table>
<thead>
<tr>
<th>Neuropsychological test</th>
<th>Blurring Mean (range)</th>
<th>No blurring Mean (range)</th>
<th>Controls Mean (range)</th>
<th>Blurring versus no blurring*</th>
<th>Blurring versus controls*</th>
<th>No blurring versus controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raven coloured progressive matrices</td>
<td>28.82 (21–35)</td>
<td>32.31 (28–36)</td>
<td>32.79 (20–36)</td>
<td>U = 51, $P = 0.01$</td>
<td>U = 111, $P = 0.001$</td>
<td>NS</td>
</tr>
<tr>
<td>Word fluency on phonemic cue</td>
<td>26.35 (13–46)</td>
<td>25.92 (12–45)</td>
<td>39.45 (21–65)</td>
<td>NS</td>
<td>U = 113, $P = 0.001$</td>
<td>U = 94, $P = 0.003$</td>
</tr>
<tr>
<td>Word fluency on semantic cue</td>
<td>32.18 (19–45)</td>
<td>33.46 (19–46)</td>
<td>42.45 (14–57)</td>
<td>NS</td>
<td>U = 95.5, $P &lt; 0.001$</td>
<td>U = 94, $P = 0.003$</td>
</tr>
<tr>
<td>Short story</td>
<td>9.29 (3–19.5)</td>
<td>10.92 (4.5–19)</td>
<td>15.61 (9–24.5)</td>
<td>NS</td>
<td>U = 82, $P &lt; 0.001$</td>
<td>U = 94.5, $P = 0.003$</td>
</tr>
<tr>
<td>Selective reminding procedure</td>
<td>91.53 (13–169)</td>
<td>118.27 (0–163)</td>
<td>125.46 (28–166)</td>
<td>NS</td>
<td>U = 115, $P = 0.019$</td>
<td>NS</td>
</tr>
<tr>
<td>Rey complex figure delayed reproduction</td>
<td>15.44 (8–25)</td>
<td>18.98 (7.32–28.72)</td>
<td>20.81 (9–32)</td>
<td>NS</td>
<td>U = 164.5, $P = 0.008$</td>
<td>NS</td>
</tr>
<tr>
<td>Corsi blocks supraspan learning</td>
<td>16.73 (6.18–27.84)</td>
<td>20.45 (7.32–28.72)</td>
<td>25.70 (18.42–18.94)</td>
<td>NS</td>
<td>U = 47.5, $P &lt; 0.001$</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = not significant; *Mann–Whitney tests.
excess of single white matter neurons was frequently observed in all samples, and their density was estimated in both groups. We found a wide range of white matter neuronal density using both 2D (30–94 neurons/mm² in Group 1 and 21–59 neurons/mm² in Group 2) and 3D cell-counting techniques (1777–3504 neurons/mm³ in Group 1 and 1636–4243 neurons/mm³ in Group 2), but no significant differences in the average density between the two groups was observed \((P = 0.22\) and \(P = 0.54\), Supplementary Fig. 1E and F). Blood vessels and, when present, perivascular spaces were intensely stained by fibrinogen immunoreactivity in all samples from both groups (Supplementary Fig. 1G and H).

**Correlative 7 T magnetic resonance imaging and ultrastructural study**

Unlike the in vivo T2-weighted images (Fig. 2A and D), there was a clear border between the grey and white matter in all of the samples (Figs 2B and E, 3A and I and 4A). However, signal intensity in the white matter was dishomogeneous in the Group 1 samples, with patchy areas of hyperintensity mainly in the depth (Figs 2B, 3A and I), whereas visual analysis of the Group 2 images showed a homogeneous hypointense signal throughout the white matter (Figs 2E and 4A). The fractional anistropy maps obtained from the same specimens showed similar grey matter values in both groups, whereas the white matter fractional anistropy values were lower in Group 1 (compare Fig. 2C with Fig. 2F). The mean diffusivity values did not reveal any clear between-group differences in either the white matter or the grey matter (data not shown).

After the imaging study, the specimens underwent morphological/ultrastructural study. The Group 1 sections processed for Black-Gold® II revealed dishomogeneous staining in the white matter, whereas the same staining was homogeneous in the Group 2 sections (compare Fig. 3B, C, E, J, K and M with Fig. 4B and C). Analysis of the adjacent semi-thin sections counterstained with toluidine blue confirmed this difference (compare Fig. 3D, F, L and N with Fig. 4D). The qualitative ultrastructural examination showed that the samples from Group 1 (particularly those from the light zone) had more extra-axonal space, fewer myelinated and unmyelinated axons, which also varied considerably in size and morphology in comparison with the Group 2 samples (compare Fig. 3G, H, O and P with Fig. 4E and F). Gial cells, glial processes and vacuoles of different size were also observed in the neuropil. On the contrary, the specimens of both groups showed no alterations in myelin sheath thickness in relation to axonal diameter and no alterations in the vessels walls. In particular, there were no desmosome alterations in the capillary endothelial cells and no alterations in the elastic and muscular tunica of the arteriolar walls. Quantitative analyses confirmed that the number of axons and axonal density in the white matter were significantly reduced in both the light and dark zones of the Group 1 samples, with no differences between the zones (Table 3). Moreover, the myelinated axons were less circular and more elongated than those in the Group 2 samples, thus indicating a tendency towards tilted axons, and their circumference was larger. The cumulative area occupied by axons represented a smaller fraction of the entire area of the Group 1 samples (particularly in the light zone), thus leading to a larger extra-axonal area (86% and 83% in the light and dark zones of the Group 1 samples, respectively, versus 79% of the Group 2 samples).

**Discussion**

The MRI features of the anterior temporal signal abnormalities commonly called ‘blurring’ consist of a less marked definition of the grey/white matter border, frequently coupled with the apparent shrinkage of the white matter core of the temporal pole. They have long been described in a subgroup of patients with temporal lobe epilepsy, and the prevalence of ipsilateral anterior temporal abnormalities in patients with intractable temporal lobe epilepsy and hippocampal sclerosis has been estimated as being 32–66% (Choi et al., 1999; Meiners et al., 1999; Mitchell et al., 1999) in adults and 57% in children (Mitchell et al., 2003). During the past 10 years, numerous attempts have been made to assess the aetiopathogenesis of temporo-polar blurring, but none of the various hypotheses has been confirmed, which means that although the MRI appearance may reflect a change in the water content of the temporal lobe, the pathological substrate of the change is still unknown. We used different methodological approaches to collect robust evidence that the blurring is caused by fibre bundle degeneration and will discuss here the morphological data and their possible clinical implications separately.

**Temporo-polar blurring and white matter fibre degeneration**

It has been suggested that temporo-polar blurring is due to vasculometabolic disturbances in the temporal lobe that are presumably secondary mechanisms of repetitive seizures (Ryvlin et al., 1998; Weder et al., 2006). Further, Chassoux et al. (2004) found that temporal hypometabolism was more extended in patients with temporo-polar signal changes. We therefore assumed that, if the blurring is due to functional vasculometabolic mechanisms, it would no longer be visible on magnetic resonance images of surgically removed and fixed temporal lobe samples; however, we found that signal alterations could still be seen in our Group 1 specimens.

Nevertheless, there were some differences between the presurgical 1.5T magnetic resonance images and the 7T images of the surgical specimens. In Group 1, the grey/white matter border was clearly marked, but signal alterations were particularly pronounced in the deep white matter; furthermore, the hyperintense signal was frequently dishomogeneous and there were patchy areas of different intensities. The differences between the 1.5 and 7T images may be due to the higher resolution of the latter (which is also due to the number of repetitions allowing the use of thinner sections) or to the fixation procedures. However, the second hypothesis can be ruled out because we have previously shown that fixation procedures do not substantially alter 7T images, which are comparable with those obtained in vivo using a 1.5T machine (Garbelli et al., 2011).
Figure 3 Correlative data from the 7 T magnetic resonance, histochemical and ultrastructural images of two patients with blurring. The dishomogeneous signal intensity in the white matter revealed by the T$_2$-weighted images (A and I) correlates with the dishomogeneous staining of the myelinated fibres clearly visualized by means of Black-Gold$^{18}$ (B and J). The high-magnification images C, K, E and M show the distribution of the myelinated fibres in a lightly stained zone (yellow asterisks) and an intensely stained zone (green asterisks), respectively. Adjacent 0.5-μm semi-thin sections counterstained with toluidine blue (D, F, L and N). Electron micrographs (G, H, O and P) obtained from the lightly stained (yellow bordered panel) and intensely stained zone (green bordered panel). The ultrastructure images clearly show a reduced number of axons in all of the sampled areas (compare with Fig. 4E and F), with a normal myelin sheath and increased extra-axonal spaces with vacuoles. Scale bars: 45 μm (C, E, K and M); 25 μm (D, F, L and N); 0.6 μm (G, H, O and P).
The findings also rule out the hypothesis that the signal alterations have a vasculometabolic origin and support the hypothesis of structural morphological alterations. Some authors have suggested that blurring might be caused by abnormalities in structural development (Hardiman et al., 1988; Thom et al., 2001), but our histopathological study revealed that patients with and without blurring may have similar neuropathological features, such as the presence of a cortical dysplasia and an excess of single white matter neurons, thus excluding their potential involvement. Other explanations of blurring are dilated perivascular spaces and inflammatory changes, but Mitchell et al. (1999) found that all of these minor changes are common regardless of the presence or absence of MRI blurring. Although we did not make a similarly precise analysis, our study did not reveal any difference in inflammatory changes or any significant alterations in blood vessels.

The presence of widespread gliosis has also been considered to explain the MRI abnormalities, but no differences have been found in the density of glial cell nuclei in the temporal white matter between patients with and without blurring (Meiners et al., 1999; Mitchell et al., 1999), although our findings are in line with these reports, as they also show considerable variability in the degree of gliosis detected by means of GFAP staining within and between the groups. However, dishomogeneous myelin staining indicated clear fibre alterations that correlated with the 7 T images, thus supporting a hypothesis that has been previously proposed on the basis of the findings of neuropathological investigations (Meiners et al., 1999; Mitchell et al., 1999; Schijns et al., 2011) and modern imaging studies (Concha et al., 2009).

Meiners et al. (1999) found significantly reduced white matter staining, suggesting myelin loss in patients with MRI signal

Table 3 Quantitative analysis of the morphometric parameters performed on semi-thin and ultra-thin sections

<table>
<thead>
<tr>
<th>Evaluated parameters</th>
<th>Blurring</th>
<th>No blurring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light zone</td>
<td>Dark zone</td>
</tr>
<tr>
<td>Number of axon (OM)</td>
<td>2174 (599)</td>
<td>2234 (294)</td>
</tr>
<tr>
<td>Axonal density (axons/µm²) (OM)</td>
<td>0.117 (0.003)</td>
<td>0.120 (0.015)</td>
</tr>
<tr>
<td>Circularity (EM)</td>
<td>0.64 (0.059)</td>
<td>0.64 (0.079)</td>
</tr>
<tr>
<td>Elongation (EM)</td>
<td>2.14 (0.26)</td>
<td>2.21 (0.24)</td>
</tr>
<tr>
<td>Circumference (EM)</td>
<td>3.93 (0.84)</td>
<td>4.15 (0.83)</td>
</tr>
<tr>
<td>Area occupied by axons (µm²) (EM)</td>
<td>1055 (166)</td>
<td>1319 (185)</td>
</tr>
</tbody>
</table>

a, b versus c = P < 0.05.
d versus e = P < 0.01.
EM = electron microscopy, ultra-thin sections; OM = light microscopy, semi-thin sections.

Figure 4 Correlative 7 T magnetic resonance, histochemical and ultrastructural images of one patient without blurring. Note the homogeneous signal intensity in the white matter revealed by the T2-weighted images (A) and the homogeneous staining of the myelinated fibres obtained from a section stained with Black-Gold (B); the asterisk indicates the area shown at higher magnification in C. D, E and F show adjacent 0.5-µm semi-thin sections counterstained with toluidine blue and electron micrographs. Note the larger number of axons and fewer extra-axonal spaces in comparison with the patients with blurring (compare with Fig. 3G, H, O and P). Scale bars: 45 µm (C), 25 µm (D); 0.6 µm (E and F).
alterations, and therefore postulated that decreased myelin density in the temporal lobe of hippocampal sclerosis patients may be due to the trans-synaptic retrograde degeneration of collaterals of different axons in association bundles. Schijns et al. (2011) suggested that loss of the grey matter border may be a persistent immature appearance due to abnormal or delayed myelination during development.

Our morphological data demonstrate that the number of fibres in the subcortical white matter of the patients in Group 1 was significantly reduced and that the percentage of the area occupied by myelinated axons was smaller in comparison with Group 2 patients. Consequently, the residual fibres are less circular, more elongated and wider presumably because of a spatial rearrangement.

Interestingly, the residual myelinated fibres did not show any substantial alteration in the myelin sheath or any signs of inflammatory processes and remyelination. As demyelination is characterized by the destruction of normally formed myelin and is often followed by remyelination associated with signs of inflammation, our morphological data seem to argue against this type of pathological process and, in line with the suggestion of Schijns et al. (2011), we believe that the term ‘dysmyelination’ (characterized by the defective biosynthesis and formation of myelin sheaths) would be more appropriate in such cases. However, whatever the mechanism may be, our data demonstrate massive axonal loss and a redistribution of the remaining fibres that is similar to that observed in chronic degeneration processes.

The light and electron microscopy neuropathological data agree with the MRI findings and show decreased fractional anisotropy values in Group 1. Although volumetric MRI has demonstrated extensive white matter abnormalities in patients with temporal lobe epilepsy, it is considered a non-specific measure, and therefore, new imaging techniques such as diffusion tensor imaging have been increasingly used for the past few years to characterize white matter architecture in vivo and have provided information about the integrity and organization of fibre tracts. In particular, diffusion tensor imaging has been extensively used on the basis of the assumption that in vivo diffusion tensor imaging abnormalities reflect underlying changes in white matter microstructure. However, although data from animal models and post-mortem human brains have given insights into the histological correlates of diffusion tensor imaging parameters, there are still considerable limitations when drawing conclusions regarding the underlying microstructural features associated with human in vivo diffusion tensor imaging findings (D’Arceuil et al., 2007). Among the various proposed indices, fractional anisotropy is most frequently used to quantify the direction of diffusion and therefore the structural integrity of tissue. Decreased fractional anisotropy values indicating a loss of white matter integrity have been found in the ipsilateral temporal lobe, the cingulum, the corpus callosum and the external capsule of patients with temporal lobe epilepsy and hippocampal sclerosis (Arfanakis et al., 2002; Gross et al., 2006; Concha et al., 2007; Focke et al., 2008), but the cause of these changes is still debated, although tissue oedema, blood-brain barrier disturbances, neuronal loss and axonal demyelination have all been postulated (Margerison and Corsellis, 1966; Bernasconi et al., 1999; Sutula et al., 2003; Seidenberg et al., 2005).

In a recent study, Concha et al. (2009) found a correlation between electron microscopy and in vivo diffusion tensor imaging findings that validated diffusion tensor imaging findings as an in vivo marker of white matter pathology. Our results show that even in surgical specimens, fractional anisotropy is altered in patients with temporal lobe epilepsy and hippocampal sclerosis, thus further confirming the correlation between altered MRI signals and neuropathological white matter alterations at light and ultrastructural level. Abnormalities in structures other than the white matter bundles in the temporal pole were not detected in the routine 1.5T MRI of our patients, and so the alterations in other fibre bundles reported by other authors might have been missed. However, it needs to be emphasized that we only considered temporo-polar abnormalities and the histopathological correlations of pre- and post-surgical images. Our study confirmed that high-resolution MRI and diffusion tensor imaging can also be performed on well-preserved and fixed surgical specimens (Garbelli et al., 2011).

Clinical correlations

Although anamnestic records of epilepsy risk factors (particularly febrile seizures) have to be considered with caution, our data do not suggest any correlation between febrile seizures and the presence of white matter abnormalities, in line with previous reports (Mitchell et al., 2003; Schijns et al., 2011). As focal cortical dysplasia or heterotopic neurons in the white matter were equally distributed in both groups, they cannot be considered responsible for the temporo-polar abnormalities, which are also not associated with the type or frequency of the seizures.

Our data confirm that patients with white matter abnormalities in the temporal pole are significantly younger at the time of seizure onset than those without abnormalities. Mitchell et al. (2003) found a significant relationship between anterior temporal changes and epilepsy onset before the age of 2 years in their paediatric population, and the population described by Schijns et al. (2011) showed that a significant percentage of patients had a mean age of 2 years at onset; however, studies of adult (or mixed) populations such as ours indicate a mean age of 7–8 years at onset. The difference in age at seizure onset in the group with blurring may be due to the fact that, as stated by Mitchell et al. (2003), ‘details of the change early in the patients’ history are often more vague when obtained in adulthood and early details not well remembered’.

All of the published studies report possible myelin alterations with an early age of epilepsy onset, but our data show massive axonal loss and preserved myelin in the residual axons, which suggests an early degenerative process involving some but not all of the fibres within the temporal lobe white matter. The disappearance of selected axonal tract(s) might be secondary to a dysmyelination process caused by repetitive abnormal firing within the temporal lobe, a hypothesis that is supported by findings showing that axonal myelination at least partially depends on signals of axon origin, and that factors inducing myelin formation are mediated by electrical activity (Lubetzki and Stankoff, 2000). It is well known that major events in myelination processes occur within the first 2 years of life, even if more subtle changes
Supplementary material

Supplementary material is available at Brain online.

References


Gao W, Lin W, Chen Y, Gerig G, Smith JK, Jewells V, et al. Temporal and spatial development of axonal maturation and myelination of...


